

## REMARKS

### I. Status of the Claims

No claims are amended in this Reply. Claims 1-38 and 40-43 were previously canceled without prejudice. Applicants reserve the right to file one or more continuation applications to any canceled subject matter. Claims 39 and 44-47 remain pending.

In the non-final Office Action, all claims are rejected under 35 U.S.C. § 103 as allegedly obvious over PCT Publication WO 00/44375 to Harper, published August 3, 2000, in view of U.S. Patent 6,335,361 to Hamilton, published January 1, 2002. The Applicants submitted a Declaration Under 37 C.F.R. § 1.131 of Dr. Steven C. Zicker with the Request for Continued Examination, but the Examiner stated on page 2 of the outstanding office action that the Declaration was not persuasive, because it was not signed by both inventors. Applicants submit herewith a Declaration Under 37 C.F.R. § 1.131 of Drs. Steven C. Zicker and Karen J. Wedekind ("Declaration"). As discussed below, Applicants invented the subject matter of the claimed invention before the effective date of both Harper and Hamilton. Thus, Applicants request that the rejection be withdrawn.

### II. Declaration Under 37 C.F.R. § 1.131 of Drs. Zicker and Wedekind

Attention is respectfully drawn to the enclosed Declaration of Drs. Zicker and Wedekind, the inventors of the claimed invention. In the Declaration, Drs. Zicker and Wedekind confirm that the invention of claims 39 and 44-47 was made on a date earlier than November 3, 1999. The showing of prior invention is sufficient to antedate the Harper and Hamilton references applied against the claims, as discussed further below.

The current claims have an effective filing date of October 31, 2000, based on support for the claims in Provisional Application No. 60/244,510 filed that day, from which the current application claims priority. In particular, compared to the current specification, the provisional contains the same description of the cognitive testing, the

same description of the diet, and the same Examples 1 and 2 giving the results of the tests.

Applicants note that the effective filing date is less than one year later than the effective date of either of the Hamilton or Harper references. For this reason, neither reference is a bar under 35 U.S.C. § 102(b). As a consequence, the references can be removed by a showing of prior invention.

The Examiner's attention is also drawn to prosecution in serial no. 11/154,210 before Examiner Simmons. There, a similar Rule 131 declaration has been submitted to antedate similar applied references. In particular, sections 5-14 of the current Declaration are largely taken from the '210 declaration.

The enclosed Declaration of Drs. Zicker and Wedekind demonstrates that the invention was made earlier than November 3, 1999. Conception of the invention is evidenced by process formulation sheets printed earlier than the effective date and corroborated by the initials of a plant manager earlier than the effective date. Appendices A, B and C and the inventors' comments demonstrate that a composition containing the four antioxidants recited in the claims was made earlier than the effective date. Appendices D and E and the inventors' comments demonstrate that the inventive concept of feeding the antioxidant diet to aged pets was also conceived before the effective date of November 3, 1999.

In particular, sections 15-21 discuss Appendix D, which gives evidence of conception of the invention and plans for testing at the Lovelace Respiratory Research Institute ("LRRRI"). As explained in the Declaration, Appendix D is dated earlier than November 3, 1999 and shows the inventors had proposed to test dog diets having the four claimed antioxidants (vitamin E, vitamin C, carnitine, and lipoic acid) in order to prevent cognitive decline. As explained in the Declaration, prevention of cognitive decline encompasses the claimed methods of inhibiting the loss of learning ability or increasing the learning ability of an aged companion pet in need of such treatment.

The discussion of Appendix E in sections 22-26 conclusively demonstrates the inventors were in possession of the claimed method before the critical date.

Specifically, Appendix E shows that the inventors had settled on a formula that contained all four antioxidants for use in the claimed method.

Conception of the invention as shown in Appendices D and E is corroborated by Appendices A, B, and C discussed in sections 5-14 of the declaration. These appendices show that the inventors had made a dog diet having the four antioxidants in the claimed ranges. Dr. Zicker's and Dr. Wedekind's sworn testimony establishes that the composition conceived and formulated in Appendices A, B, and C was intended for animal testing at LRR1 as shown in Appendices D and E.

The touchstone of prior invention is conception. The sworn facts in Dr. Zicker's declaration unequivocally establish conception of the claimed invention prior to the effective dates of the cited references.

### III. The Hamilton Reference, US 6,335,361

The Hamilton reference has an earliest possible effective date of November 3, 1999, based on its claim of priority to Provisional Application No. 60/163,352 filed that day. For the purposes of this discussion, Applicants assume, without conceding, that the November 3, 1999 provisional has the same disclosure as the January 1, 2002 publication. The effective date of the Hamilton reference is, accordingly, less than one year earlier than the effective filing date of the current claims. Thus, the Hamilton reference is not a bar under 35 U.S.C. § 102(b); it can be removed by a showing the invention was made earlier than the reference's effective date.

Applicants respectfully submit that the Hamilton reference is effectively removed as prior art by the showing in the Declaration that the invention was made earlier than its effective date of November 3, 1999. For this reason, Applicants respectfully request the rejection over the Hamilton reference be withdrawn.

### IV. The Harper Reference, WO 00/44375

The earliest effective date of the Harper reference is its international filing date of January 31, 2000, which is less than one year before the earliest effective date of the

claims, as described above. Accordingly, the Harper reference is not a bar under 35 U.S.C. § 102(b) and as such can be removed as prior art by a proper showing of prior invention.

As demonstrated above in the discussion of the Hamilton reference, Applicants have demonstrated that they made the claimed invention earlier than November 3, 1999. Such a showing is sufficient to show the invention was made earlier than the earliest effective date of the Harper reference. Thus, the Harper reference has been removed as prior art by a showing of earlier invention.

Because Harper is not available as prior art, Applicants respectfully request the rejections using the Harper reference be withdrawn.

V. Allowable Claims 39 and 44-47

The Hamilton and Harper references applied against the claims in the Office Action of June 29, 2007 have been removed by a showing of prior invention. There being no other rejections of record, Applicants believe that the claims are now in an allowable condition and respectfully request an early Notice of Allowance.

The Examiner is invited to telephone the undersigned if that would be helpful to resolving any issues.

Respectfully submitted,

Date: November 13, 2008

By: Shannon E. McGarrah  
Shannon E. McGarrah  
Reg. No.: 55,442  
COLGATE-PALMOLIVE COMPANY  
909 River Road; P.O. Box 1343  
Piscataway, NJ 08855-1343  
Telephone: (732) 878-7151

# EXHIBIT A

# CONFIDENTIAL

Hill's® Pet Nutrition, Inc. - Science and Technology Center

## Make Sheet for Grain Mix

For production at

Requested by Steven Zicker

Product Number:  
Formula Number: 16668  
Variable Number:  
Project Number:  
Job Code:

Product Name:  
Formula Description: NMSENIOR TEST+CARNIT/TAU  
Variable Description:  
Project Description:  
Job Code Description:

Total lbs finished product: 6,000.00  
Process % of finished product: 94.85  
Pounds from this process: 5,679.18  
Pounds per batch: 1,893.06  
Batches required: 3.00  
Flow rate from this process: 0.00

### Make Sheet Comments:

Package in 40 lb white bags  
Cheryl to provide labels  
Ship to New Mexico/Toronto as per Cheryl  
Save 1x40lb bag for analysis

### Ingredient Listing

Ingr Code	Ingredient	% of Diet	% of Mix	Total lbs	lbs Batch
2121.4	Yellow Corn, Whole	62.485	66.015	3749.100	1249.70
2322.4	P.Meal RA Perdue	11.105	11.732	666.300	222.10
2155.4	Rice, Brewers Milled	5.000	5.282	300.000	100.00
2175.4	Soybean Mill Run	4.500	4.754	270.000	90.00
2124.4	Corn Gluten Meal	2.600	2.747	156.000	52.00
2193.1	Egg, Compacted	1.000	1.056	60.000	20.00
9483.1	Spinach Fl LaBud	1.000	1.056	60.000	20.00
9485.1	Citrus pulp, Trp	1.000	1.056	60.000	20.00
9486.1	Tomato Pomace WE	1.000	1.056	60.000	20.00
9487.1	Grape Pomace ACIR	1.000	1.056	60.000	20.00
9488.1	Carrot Dr LaBud	1.000	1.056	60.000	20.00
2103.4	Flaxseed, Ground	0.600	0.634	36.000	12.00
PRE	Grain Premix	2.363	2.496	141.780	47.26
TOTALS		94.653	100.000	5679.18	1893.06

1-16.7 lbs  
RE Stock 2,

LS+ [REDACTED]  
[REDACTED]  
[REDACTED]

Approved: \_\_\_\_\_ Date: \_\_\_\_\_

# EXHIBIT B

# CONFIDENTIAL

Hill's Pet Nutrition, Inc. - Science and Technology Center

## Make Sheet for Grain Premix

For production at

Requested by Steven Zicker

D  
AR

X135

Inline Enrobe

collect As much  
as possible

Product Number: 100219

Formula Number: 16668

Variable Number: 0307

Project Number: 100219

Job Code: 0307

Product Name: NMS 2-45 T-5

Formula Description: NMSSENIOR TEST+CARNITITAU

Variable Description:

Project Description:

Job Code Description:

Total lbs finished product: 6,000.00  
Process % of finished product: 2.36  
Pounds from this process: 141.78  
Pounds per batch: 47.26  
Batches required: 3.00  
Flow rate from this process: 0.00

### Make Sheet Comments:

Package in 40 lb white bags  
Cheryl to provide labels  
Ship to New Mexico/Toronto as per Cheryl  
Save 1x40lb bag for analysis

### Ingredient Listing

Ingr Code	Ingredient	% of Diet	% of Mix	Total lbs	lbs Batch
2282.4	Potassium Chloride	0.464	19.636	27.840	9.28 ● ● ●
2208.4	Dicalcium Phosphate	0.368	15.573	22.080	7.36 ● ● ●
2271.4	Choline Chloride, 60%	0.260	11.003	15.600	5.20 ● ● ●
9624.4	Carotene 10% NF	0.257	10.876	15.420	5.14 ● ● ●
9287.1	VITAMIN E 50 ADS	0.241	10.199	14.460	4.82 ● ● ●
2261.4	Myrplex 600P Glyceryl Monooleate	0.200	8.464	12.000	4.00 ● ● ●
2269.4	Salt, Iodized	0.179	7.575	10.740	3.58 ● ● ●
2201.4	Calcium Carbonate	0.100	4.232	6.000	2.00 ● ● ●
2352.4	Vit 2352-49 dry	0.065	2.751	3.900	1.30 ● ● ●
2206.4	Taurine	0.053	2.243	3.180	1.06 ● ● ●
9061.1	L-Tryptophan	0.044	1.862	2.640	0.88 ● ● ●
2305.4	2305 Mineral Mix	0.042	1.777	2.520	0.84 ● ● ●
2237.1	Ascorbic Acid <u>Coated (EC)</u>	0.029	1.227	1.740	0.58 ● ● ●
9429.1	Naturax Plus Dry	0.020	0.846	1.200	0.40 ● ● ●
2207.1	Magnesium Oxide (MAGOX)	0.017	0.719	1.020	0.34 ● ● ●
9517.1	α-lipoic acid	0.014	0.592	0.840	0.28 ● ● ●
9516.1	So-Yeast	0.010	0.423	0.600	0.20 ● ● ●
TOTALS		2.363	100.000	141.78	47.26

Stay-C 35%

.029 1.227 1.740

0.58 ● ● ●

Approved: \_\_\_\_\_ Date: \_\_\_\_\_

LS+ [REDACTED]

KM [REDACTED]



# EXHIBIT C

# CONFIDENTIAL

Hill's® Pet Nutrition, Inc. - Science and Technology Center

## Make Sheet for Topical

For production at

Requested by Steven Zicker

Product Number:  
Formula Number: 16668  
Variable Number:  
Project Number:  
Job Code:

Product Name:  
Formula Description: NMSENIOR TEST+CARNIT/TAU  
Variable Description:  
Project Description:  
Job Code Description:

Total lbs finished product: 6,000.00  
Process % of finished product: 5.35  
Pounds from this process: 320.82

### Make Sheet Comments:

Package in 40 lb white bags  
Cheryl to provide labels  
Ship to New Mexico/Toronto as per Cheryl  
Save 1x40lb bag for analysis

### Ingredient Listing

Ingr Code	Ingredient	% of Diet	% of Mix	Total lbs	lbs Batch
2179.4	Soybean Oil, Crude Degummed	2.247	42.024	134.820	798.53 66.95
8123.4	Grease, Choice White	2.100	39.274	126.000	249.45 43.00
8271.4	Optimizer LDPE H-Plus	1.000	18.702	60.000	354.04 20.00
TOTALS		5.347	100.000	320.82	1893.66 106.95

Approved: \_\_\_\_\_ Date: \_\_\_\_\_

# EXHIBIT D

# APPROVED COPY

1

Refer to Instructions for Completing Form 8225  
This Form Must Be Typed Please Number All Pages

## For Office Use Only

Three-year  
Approval Period:

Date Received  
Protocol/Proposal#  
Approval Date

Lovelace Respiratory Research Institute  
Institutional Animal Care and Use Committee  
PROTOCOL/PROPOSAL TO USE VERTEBRATE ANIMALS IN RESEARCH, TESTING,  
OR INSTRUCTION  
(Form 8225)

☒ New Protocol/Proposal  
☐ Renewal Protocol/Proposal;  
(Previous Protocol/Proposal # \_\_\_\_\_.)

Principal Investigator/Study Director  
(Must be a Staff Member or Postdoctoral Fellow) Bruce A.  
Muggenburg

Title: Senior Scientist

Division/Unit Administering Accounts: Toxicology

Mailing Address: \_\_\_\_\_ North ☒ South \_\_\_\_\_ Other

Telephone # (505) 845-1119 FAX # (505) 845-1198

E-mail address: bmuggenb@lrri.org

Other Contact Person(s):

Name: F. F. Hahn Position: Study Pathologist

Name: Position:

Project/Title: (Enter Same Title as Grant Applications)

Title: The effects of Hill's antioxidant diet on the development  
of age-dependent cognitive dysfunction and neuropathology in  
canines.

Funding Agency: Hill's Pet Nutrition Currently Funded?  
Yes \_\_\_\_\_ No ☒

Present Grant/Contract Number (if known):

To which account should Animal Care charges be billed to?

SUBMIT COMPLETED FORM TO: INSTITUTIONAL ANIMAL CARE & USE  
COMMITTEE, ATTN: -IACUC-Office, LRRI SOUTH. TELEPHONE: 845-1025.  
ALLOW 2-4 WEEKS FOR APPROVAL.

# APPROVED COPY

2

## DESCRIPTION OF PROJECT (QUESTIONS 1 - 5)

1. Which of the following describes the type of animal use proposed in this application? (Check all that apply.)

☒ Basic Research  
☐ Applied Research  
☐ Field Research  
☐ Instruction or Training  
☐ Testing (toxicology, etc.)  
☐ Service (breeding, core facility, surveillance)  
☐ Other (Specify \_\_\_\_\_)

2. How would you explain to a non-scientist the long-term or overall scientific goals of the proposed work?

Pet owners and veterinarians have long recognized that some older dogs develop behavior changes suggesting senility. Research done at this Institute and elsewhere have shown that dogs do develop age-dependent dementia and that the neuropathology and loss of memory and learning skills are similar to these changes in aged humans. Because the dog appears to be a good model for human brain aging, we propose to evaluate two interventions that may delay the onset of age-dependent dementia. The two interventions are: 1) a diet high in antioxidants in the form of Vitamins E and C, and food products with naturally occurring anti-oxidants, and 2) a program of enriched learning and physical exercise experiences.

3. How would you explain to a non-scientist the specific objectives of the proposed work?

The dogs will be divided into four groups. The objective for each group is given below:

1. Twelve dogs will be in the control group and will be fed a normal balanced diet. Each dog will be housed individually in a run and will be evaluated for cognitive function and physical health once a year. This group is expected to have normal aging.
2. Twelve dogs will be in the antioxidant diet group. These dogs will be treated like the dogs in group 1 but will receive the diet rich in antioxidants (Vitamins E and C, etc.). This group is expected to age with smaller losses in learning and memory abilities than the dogs in group 1.
3. The twelve dogs in this group will receive the normal balanced diet but will be housed with a pen mate and will be given mental and physical enrichment. We hypothesize this group will have smaller losses in learning and memory than group 1 dogs because exercise has been shown to have an antioxidant effect.
4. The twelve dogs in this group will receive the high antioxidant diet and the mental and physical enrichment. We expect this group to have significantly smaller aging changes than any of the other groups.

(This study is one half of a larger study. Only 24 dogs are in this study and the other 24 dogs are in protocol FY98-001.)

5. How would you explain to a non-scientist the ways the proposed animal use might benefit human or animal health or the environment,

## APPROVED COPY

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the advancement of knowledge, or the good of society?

This study will test the hypothesis that at least part of the aging process is due to oxidant damage to the brain, damage that leads to the death of neurons and the subsequent loss of memory and learning functions. If these interventions prove successful over the course of this study, it will provide a way in which both humans and their companion animals (at least dogs) can improve the quality of life during aging.

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4

The IACUC Office is required to prepare a list of experimental or instructional procedures performed on animals for the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

5. From the list below, check all experimental or instructional procedures that will be performed on the animals requested in this application. All procedures checked below must be explained in Question 8. (Check all that apply.)

- |   |  |
|---|--|
| <input type="checkbox"/> Addiction or addiction withdrawal          | <input type="checkbox"/> Irradiation   |
| <input type="checkbox"/> Amputation                                 | <input type="checkbox"/> Irritation, experimental                                    |
| <input checked="" type="checkbox"/> Anesthesia                      | <input type="checkbox"/> Lavage  |
| <input type="checkbox"/> Antibody production                        | <input type="checkbox"/> Myocardial infarction                                       |
| <input type="checkbox"/> Ascites production                         | <input type="checkbox"/> Noxious stimulus  |
| <input type="checkbox"/> Behavior modification/operant conditioning | <input type="checkbox"/> Obesity, experimental                                       |
| <input type="checkbox"/> Biopsy                                     | <input type="checkbox"/> Observation only (no manipulations)                         |
| <input checked="" type="checkbox"/> Blood collection                | <input type="checkbox"/> Organ/system failure or dysfunction, experimentally induced |
| <input type="checkbox"/> Breeding                                   | <input type="checkbox"/> Paralysis, experimentally induced                           |
| <input type="checkbox"/> Burn                                       | <input type="checkbox"/> Peritoneal lavage   |
| <input type="checkbox"/> Cannulation                                | <input type="checkbox"/> Prey, animal  |
| <input type="checkbox"/> Catheterization                            | <input checked="" type="checkbox"/> Radiography                                      |
| <input type="checkbox"/> Capture of wildlife                        | <input type="checkbox"/> Restraint   |
| <input type="checkbox"/> Cardiac puncture                           | <input type="checkbox"/> Sensory dysfunction   |
| <input type="checkbox"/> Dental procedure                           | <input type="checkbox"/> Sepsis induction  |
| <input checked="" type="checkbox"/> Environmental manipulation      | <input type="checkbox"/> Stress  |
| <input checked="" type="checkbox"/> Euthanasia                      | <input type="checkbox"/> Stroke  |
| <input checked="" type="checkbox"/> Food/water manipulation         | <input type="checkbox"/> Surgical procedure, non-recovery                            |
| <input type="checkbox"/> Gavage                                     | <input type="checkbox"/> Surgical procedure, recovery                                |
| <input type="checkbox"/> Immunization, experimental                 | <input type="checkbox"/> Toxicity test   |
| <input type="checkbox"/> Immunosuppression                          | <input type="checkbox"/> Transplantation   |
| <input type="checkbox"/> Implant                                    | <input type="checkbox"/> Tumor growth, experimentally induced                        |
| <input type="checkbox"/> Injury/trauma                              | <input checked="" type="checkbox"/> Other <u>Cognitive testing</u>                   |
| <input type="checkbox"/> Injection                                  |  |
| <input type="checkbox"/> Inhalation exposure                        |  |
| <input type="checkbox"/> nose-only whole body                       |  |
| <input type="checkbox"/> Inoculation, experimental                  |  |

LOVELACE RESPIRATORY RESEARCH INSTITUTE  
ANIMAL CARE AND USE COMMITTEE PROTOCOL DISPOSITION FORM

Protocol/Proposal #: [REDACTED]

Investigator: B. A. Muggenburg

Review Date: [REDACTED]

☐ Proposal XX New Protocol ☐ Annual Review ☐ Three Year Resubmittal ☐ Other

Dear Dr. B. A. Muggenburg:

Your protocol, entitled, "The Effects of Hill's Antioxidant Diet on the Development of Age-Dependent Cognitive Dysfunction and Neuropathology in Canines", was considered by the Institutional Animal Care and Use Committee, and the following decisions were made:

☒ The proposal/protocol/amendment\*\* was approved as presented.

☐ The protocol/proposal was not approved by the committee for reasons stated in the attached memorandum.

☐ The annual report was approved for another year.\*\*

☐ The annual report was accepted and completion of the study noted. The file has been inactivated.

☐ The contingencies outlined by the committee in its initial review have been satisfied and the protocol has received final approval.\*\*

☐ The committee recognizes no major problems or unacceptable practices in the protocol/proposal, however, some changes are needed or additional information is requested. The protocol can be approved when answers to the committee's questions or modifications to the protocol listed on the attached memorandum are received and approved by the chairman or his designee.\*\*

☐ The committee has serious questions and/or concerns about the research protocol. These are described in the attached memorandum. The amendment to the protocol cannot be approved until the committee reviews the response to the questions and/or concerns.

If you have any questions you may contact Dr. David Burt (845-1018) or Dr. Steve Rohrer (845-1049) and discuss your protocol.

Thank you,

David Burt

Date: [REDACTED]

Stephen R. Rohrer, PhD, Chairman, IACUC  
or \*\*David G. Burt, DVM, Attending Veterinarian



# EXHIBIT E

## Table of Contents

### Introduction

The purpose of the current research project is to determine the effects of both antioxidants and environmental enrichment on age-dependent cognitive decline in a 3 year longitudinal design using beagle dogs. Dogs will undergo baseline screening of cognitive function and general health evaluation including clinical pathology and physical examinations. Magnetic resonance scans will be used to obtain in vivo measures of brain and cerebrovascular function. Each dog will be placed into one of four groups, which are counterbalanced with respect to cognitive ability, sex and age: (1) control group (2) enriched environment (3) dietary enrichment and (4) combined dietary and environmental enrichment. A broad spectrum of antioxidants will be added for dietary enrichment. The environmental enrichment condition will consist of additional cognitive experience and enriched sensory environment. Cognitive function, physical health and brain MR's will be monitored yearly to establish ongoing effects of the treatment. At the end of the study, detailed histological analysis of brain tissue will be correlated with cognitive function and MR measures of brain atrophy and cerebrovascular function to establish the effectiveness of the treatments on delaying or preventing the development of age-dependent neuropathologies.

### Progress Report

In year 1 we proposed to collect baseline measures and to begin the intervention studies. Each of the baseline measures will be discussed separately below.

#### *Evaluation of Health Status*

The physical and neurological examinations for individual dogs have been completed and the general health of the dogs indicates no illnesses, sensory or motor impairments that exclude participation in the study.

Blood biochemistry profiles indicate that most dogs fall within the range of values considered normal for 51 individual blood biochemistry measures. No significant differences between the blood biochemistry profiles across treatment groups are evident except for corrected calcium levels indicating that dogs assigned to the enriched environment and antioxidant combination group have lower levels than the other 3 groups ( $F(3,21)=3.387$   $p<.041$ ). However, considering the number of comparisons being made across the 51 measures, it is more than likely that this is a spurious difference.

#### *Spontaneous Behavior Tests*

Baseline testing of open field behavior is complete and includes measures of distance traveled, urination frequency, sniffing frequency, rearing and jumping frequency, time spent inactive and vocalization frequency. An analysis of variance comparing the 4 groups on these baseline values indicate no significant differences across treatment groups as shown in Table 1.

TABLE 1. ANOVA OF OPEN FIELD BEHAVIORS

		df	F	Sig.
DISTANCE	Between Groups	3	.173	.913
	Within Groups	20		
	Total	23		
URINE	Between Groups	3	.882	.467

	Within Groups	20		
	Total	23		
SNIFF	Between Groups	3	1.690	.201
	Within Groups	20		
	Total	23		
INACTIVITY	Between Groups	3	1.521	.240
	Within Groups	20		
	Total	23		
GROOM	Between Groups	3	1.319	.296
	Within Groups	20		
	Total	23		
REAR	Between Groups	3	2.216	.118
	Within Groups	20		
	Total	23		
VOCAL	Between Groups	3	2.036	.141
	Within Groups	20		
	Total	23		
JUMP	Between Groups	3	1.143	.356
	Within Groups	20		
	Total	23		

#### *Pretraining in testing apparatus*

**Reward Approach Learning.** This task is intended to be a pretraining phase to teach dogs to work in the test apparatus. Dogs are taught to look for a food reward in one of 3 recessed food wells rather than to rely upon olfactory cues. Dogs are given 10 trials per day and are required to meet one of two criteria: 9/10 correct on one day or 8/10 correct on 2 consecutive days. The score assigned to individual animals is the sum of errors across all days of testing up to and including the day when criterion was met. Of 28 dogs originally screened, 24 dogs were able to learn the reward approach task and will be used for the long term study. No significant group differences were noted on this measure of skill-learning (Table 2)

**Object Approach Learning.** This task is used to train dogs to manipulate objects on the presentation tray in the test apparatus. As reward approach learning, no significant differences were found across the four treatment groups at baseline (Table 2).

**Visual Discrimination Learning.** This task measures habit formation in dogs and involves presenting dogs with two objects simultaneously. Only one of these objects is consistently associated with a food reward. For dogs to be able to learn this problem they must also have sufficient visual acuity to differentiate the two objects and ensures that sensory ability is not compromised in individual dogs. No significant differences across treatment groups on this task were found (Table 2)

**Reversal Learning.** This task proceeds in an identical manner as visual discrimination learning, using the same 2 objects, however, the reward contingencies are reversed. Dogs are required to inhibit a response to the previously correct object and to learn to respond to the previously incorrect object. All dogs were able to learn the task and no significant group differences on this baseline measure were found (Table 2).

#### *Training on the Spatial Memory Test*

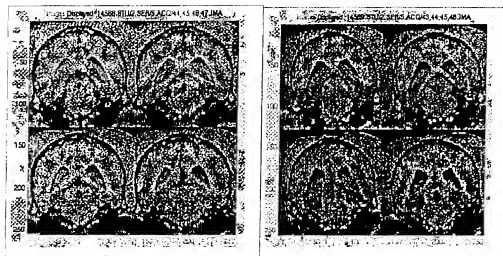
All dogs have completed testing on a spatial delayed non match to position task and found the problem particularly difficult to learn. Four of 24 dogs were able to meet criterion and no significant differences exist between the four treatment groups (Table 2).

#### *Training on the Object Recognition Memory Test*

All dogs have completed testing on an object recognition non match to sample task and 15 of 24 dogs were able to learn the problem. Of the 15 dogs that met criterion, some dogs were able to remember information for as long as 150 seconds. No significant differences exist between the four treatment groups (Table 2).

#### *Baseline Magnetic Image Resonance Scans*

Baseline MRI's have been conducted on all 24 dogs. All dogs tolerated the procedures well and suffered no adverse side effects. The figure below illustrates coronal sections from 2 dogs that are typical of each treatment groups. MR scans indicate no gross abnormalities in any of the dogs included in the study. The anatomical and cerebrovascular analyses are underway and are being conducted by Dr. Lydia Su at the University of California as per our protocol. Procedures are being standardized to be applied to individual dogs and across scanning sessions.



*Figure 1. MR images are presented for two representative dogs illustrating no gross abnormalities. Note that the dog on the right appears to have larger ventricles than the dog on the left, which will be quantified using volumetric analyses.*

**Group Assignments:** To balance the treatment groups, the error scores from individual dogs were summed across all of the baseline cognitive tasks (reward and object approach learning, visual discrimination and reversal, object and spatial memory). Males and females were split into 2 groups and dogs were given a ranking from 1 to 12 based upon an ascending series of the individual error scores. Dogs were assigned to each group counterbalanced for sex and for rankings on the cognitive test scores. The table below illustrates that placing dogs in treatment groups in this manner results in equally matched groups of dogs across all tasks, illustrated by the lack of significant F values for all tasks.

TABLE 2. ANOVA FOR TESTING FOR GROUP DIFFERENCES IN BASELINE COGNITIVE TEST SCORES

		Sum of Squares	df	Mean Square	F	Sig.
Reward Approach	Between Groups	207.792	3	69.264	.826	.495
	Within Groups	1677.167	20	83.858		
	Total	1884.958	23			
Object Approach	Between Groups	10.792	3	3.597	.548	.655
	Within Groups	131.167	20	6.558		
	Total	141.958	23			
Visual Discrimination	Between Groups	928.833	3	309.611	2.137	.127
	Within Groups	2897.000	20	144.850		
	Total	3825.833	23			
Reversal Learning	Between Groups	2981.667	3	993.889	1.617	.217
	Within Groups	12295.667	20	614.783		
	Total	15277.333	23			
Object Recognition Memory	Between Groups	4374.792	3	1458.264	.191	.901
	Within Groups	152307.833	20	7615.392		
	Total	156682.625	23			
Spatial Memory	Between Groups	21337.518	3	7112.506	2.209	.122
	Within Groups	57965.300	20	3220.294		
	Total	79302.818	23			

Exercise and Environmental Intervention: All dogs are now either housed singly or in pairs depending upon the experimental condition. Dogs have begun an exercise program that involves walking on a leash for period of 20 minutes, twice per week. The first series of additional learning experiences is underway with dogs being trained on a landmark discrimination task. Dogs are being tested 5 times per week.

Antioxidant Diet Intervention: A commercial, senior formula, dog food was selected for the basal diet. The basal formula meets all AAFCO (American Association of Feed Control Officials) recommendations for a food to be fed to domestic dogs. A variety of ingredients were considered for fortification of the diet.

Raw ingredients considered were screened for carotenoid, flavinoid, and ORAC (oxygen radical absorbance capacity) content. From these data the 5 ingredients with the highest combined ORAC/carotenoid/flavinoid content that were economically & legally feasible for inclusion into a commercial feed were selected. The components selected were: spinach, carrot granules, grape pomace, tomato pomace, and citrus pulp. Each of these was included at a 1% inclusion rate into the diet as a direct substitute for corn.

Purified or synthetic ingredients considered for inclusion were: vitamin C (Stay-C [Roche]), vitamin E acetate, beta-carotene, carnitine, and lipoic acid. Selenium yeast was also included since selenium bioavailability in common ingredients for pet foods is low (20-30%) A range for the rate of dietary inclusion was extrapolated from other mammalian studies based on dose per body weight or dose per body weight to the  $\frac{1}{4}$  power. In the case where insufficient data in other species existed to convert by body weight a best estimate was made.

#### Experimental foods

Variations of the basal diet were made based on the above ingredients. All numbers are in ppm per dry matter of food.

**Table 3. Targeted vitamin level additions in antioxidant diets**

Treatments	Vit E	Vit C	$\beta$ -carotene	Selenium	Flavonoids	Carotenoids	Lipoate
1.Ctrl	50	—	—	.6		X	
2.half vit.	250	100	6	.66		X	
3.full vit w/o lipoate	500	200	12	.66		X	
4.full vit + frt/veggie	500	200	12	.66	X	X	
5.fruit/veggie only	50	30	0.2	.6	X	X	
6.full vit w/ lipoate	500	200	12	.66		X	100

These 6 diets were then put into a feeding trial that utilized 48 dogs (8 dog per group) at Hills Pet Nutrition in house. All dogs were fed basal diet for 4 weeks prior to intervention with food. A control group was maintained on basal diet for the duration of the study. Dogs were blocked into groups based on age and sex. Dogs were fed intervention diets for 4 weeks.

**Table 4. Mean post-antioxidant measurements as affected by diet.**

Diet	ORAC- total	Sera Vit E	Sera Vit C	Urinary isoprostane -Cr	Urinary 8- OH-dG-Cr
	$\mu\text{M}$	$\mu\text{g/ml}$	$\mu\text{g/ml}$	ratio	ratio
1. Senior control	3683	58.9	20.7	20.8	.34
2. Senior + half vitamin	4327	70.8	20.1	16.8	.64
3. Senior + full vitamin	4726	92.1	17.5	21.3	.45
4. Senior + full vitamin + fruit/veg	4447	88.2	46.8	19.7	.42
5. Senior + fruit/veggie only	4037	61.9	32.6	27.5	.44
6. Senior + full vitamin + lipoate	5066	94.2	28.1	15.3	.25
<b>Typical ranges for humans:</b>	<b>3300-5000</b>	<b>6.5 - 17.2</b>	<b>7 - 26.5</b>	<b>0-34</b>	<b>0-0.74</b>

Based upon our findings, these data would suggest that the half and full inclusion rates of vitamins C, E, and Selenium were efficacious in improving antioxidant status as measured by changes in ORAC and sera E. Although it was our intention to evaluate the efficacy of beta-carotene in this study, because of high beta-carotene losses in the first formulations and poor dispersion in the topicals with the second round of formulations, we were unable to evaluate beta-carotene. The addition of fruits and vegetables had a positive effect on serum vitamin C concentration. The addition of lipoate to the supplemental vitamin mix trended towards significance in reducing urinary isoprostane and 8-OH-2'-deoxyguanosine. Utilizing the above information it was concluded that a mix of antioxidant ingredients and pure vitamin additions would provide the best matrix for increasing protection from free radical damage without any adverse effects in the target species. Therefore the final intervention diet was formulated to be comprised of the basal diet plus additions of the following ingredients:

1% each of carrots, spinach, tomato pomace, grape pomace, citrus pulp  
Vitamin E

Ascorbic acid as Stay-C

Selenium  
Carnitine  
Lipoic acid

#### *Efficacy of Diet Intervention*

We have collected blood samples from all the control diet dogs and 5 of the intervention diet dogs to assay vitamin E levels after 3 months of being fed the diet rich in antioxidants while on study. Dogs receiving the antioxidant diet show a significant increase in blood levels of vitamin E ( $t(5)=2.824$   $p<.048$ ).

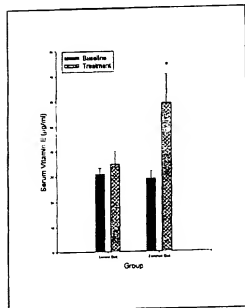


Figure 2. The group mean of dogs receiving the control diet are compared to the group mean of dogs receiving the antioxidant diet. The antioxidant diet results in a significant increase in serum levels of vitamin E (\* indicates  $p<.05$ ).

